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JOURNAL ARTICLE

Enzymatic Activities Related to the Decomposition of Coniferous Leaf Litter[1]

BRIAN P. SPALDING[2]

ABSTRACT

The rate of CO_2 evolution at 23°C and 75% moisture content for a diverse group of coniferous leaf litter samples from Oregon was measured. Significant correlations (1% level) were observed between the rate of CO_2 evolution and the activity of amylase, cellulase, and xylanase (r = 0.71, 0.58, and 0.44, respectively) in 0.05 M potassium acetate, pH 5.0 extracts of the litter. The amounts of extractable hexose, phenolics, and reducing substances were also correlated with the rate of CO_2 evolution (r = 0.74, 0.53, and 0.73 respectively). Peroxidase and invertase activities were not correlated with the rate of CO_2 evolution. Litter extracts showed zero-order kinetics for the hydrolysis of carboxymethylcellulose, xylan, starch, and sucrose. All carbohydrase activities were destroyed by a 15 min exposure to a boiling water bath.

Additional Index Words: **amylase, cellulase, xylanase, extractable hexose, rate of CO_2 evolution.**

IN ORDER TO EXPLAIN how different types of plant litter decompose at different rates under the same environmental conditions, it is necessary to examine both the organic chemical constituents of the litter and the nutritional requirements and preferences of the heterotrophic microorganisms living in the litter. Classically, the succession of microorganisms on decomposing litter has been observed (3) and the degradative processes in the litter then inferred from the physiological traits of the microorganisms in vitro (9). Other investigators (5) have centered their attention on changes in certain fractions (e.g., hemicellulose and cellulose) during the course of litter decay. This has led to the general conclusion that some fractions of plant litter, particularly lignin, are more resistant to decomposition than are cellulose, hemicellulose, and protein (17). If such generalizations are valid, then the short term, i.e. average daily, rate of litter decomposition ought to be limited by the amount of the most readily-decomposible component assuming optimum or nonlimiting environmental conditions for microbial growth. Total annual weight loss, however, would be limited by the amount of the most resistant substrate (17).

The breakdown of the many macromolecular constituents of plant litter must be extracellular and, likely, involves the agency of enzymes. Comminution of litter by the macro- and microfauna would accelerate the rate of decomposition (perhaps by increasing surface area) but the production of assimilable biochemicals would remain extracellular and enzymatic. If the hydrolysis of some litter component such as cellulose were limiting to the rate of decomposition then the amount of enzyme effecting that hydrolysis ought to be proportional to the overall rate of decomposition as measured by CO_2 evolution. In the present study, the decomposition rate of coniferous leaf litter samples from a population, diverse in botanical composition and age, was measured and the activities of several extractable enzymes were determined to observe their relationship to the measured rates of CO_2 evolution. Likely enzymes, including xylanase and cellulase, were examined because of the dominance of hemicellulose and cellulose in this coniferous leaf litter (29). Peroxidase was investigated because of its suspected role in lignin decomposition (14). Amylase and invertase, although not directly involved in coniferous litter decomposition, were examined since they have been found to be correlated with angiosperm leaf litter decomposition (21) and, on several occasions, with soil respiration (19, 24). Figure 1 summarizes a simplified conceptual model of coniferous leaf litter decomposition upon which the experimental approach of this investigation was based.

In order to assay any enzymatic activity in litter or soil, it is essential to establish the validity of the assay method. Therefore, a considerable part of the present work involves demonstrating zero-order kinetics for all the enzymatic reactions over the range of activities encountered in the litter samples. Also, since no one buffer extract would likely yield maximal activities of all the enzymes examined, several buffers were examined in order to select a good compromise; the actual pH at which an enzymatic activity is determined affects only the sensitivity and not the validity of the assay if all determinations are performed at the same pH. It is also important to demonstrate the heat lability of the catalytic activities which is good evidence that these catalysts are indeed enzymes.

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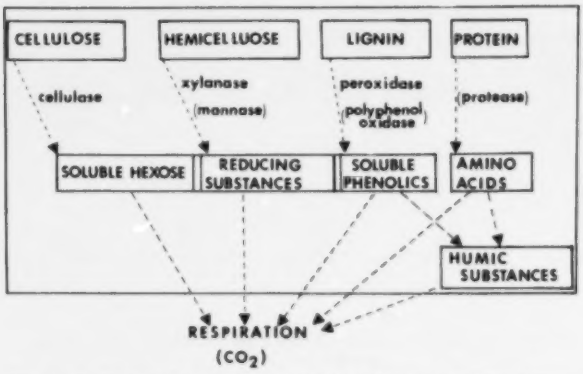

Fig. 1—A simplified conceptual model of coniferous leaf litter decomposition showing the relationship among organic litter constituents, enzymes, and respiration.

METHODS AND MATERIALS

Four areas in Oregon were selected for sampling: the H. J. Andrews Experimental Forest, Willamette National Forest, near Blue River, Oregon; Cascade Head Experimental Forest, Siuslaw National Forest, near Otis, Oregon; McDonald Experimental Forest, Oregon School of Forestry, near Corvallis, Oregon; and the Santiam Pass Area of the Willamette and Deschutes National Forests between Upper Soda and Black Butte, Oregon. Within each area, litter was sampled to represent the diversity of stand age, canopy closure, elevation, aspect, microrelief, and botanical composition. Sampling was carried out from 1 March to 7 June 1976. Among the four areas, average annual precipitation ranges from 800 to 2,500 mm and elevation from 49 to 1,400 m; all areas except higher elevations are characterized by a maritime climate-wet, mild winters and cool, relatively dry summers with a long, frost-free season. The botanical composition of each area varies (8). Large tree species in the Cascade Head sampling area are dominated by *Picea sitchensis* (Bong.) Carr. (Sitka spruce), *Tsuga heterophylla* (Raf.) Sarg. (Western hemlock), *Thuja plicata* Donn. (Western redcedar), and *Pseudotsuga menziesii* (Mirb.) Franco (Douglas fir). The McDonald area is dominated by Douglas fir and *Quercus garryana* Dougl. (Oregon white oak). Dominant species in the Andrews area are similar to the Cascade Head area, but without Sitka spruce. The Santiam area is dominated by Douglas fir and Western hemlock with some inclusions of *Pinus contorta* Dougl. (Lodgepole pine), *Pinus monticola* Dougl. (Western white pine), *Pinus ponderosa* Laws. (Ponderosa pine), *Abies amabilis* (Dougl.) Forbes (Pacific silver fir), *Abies grandis* (Dougl.) Lindl. (Grand fir), and Western red cedar.

A sample consisted of approximately 100 g of field moist litter collected to the surface of the mineral soil. Each sample was a composite of at least six handfuls of litter collected at random over a 100 m^2 area. Samples were placed in polyethylene bags and stored at 2°C until respiration measurements were initiated within 4 days of collection. Each sample was sorted by hand to remove all nonleaf material (twigs, cones, bark, and soil aggregates) and the leaf material then thoroughly mixed. Two 10 g subsamples were dried to constant weight (ca. 2 hours) at 105°C to determine moisture content. After completing the biochemical analyses, samples were dried overnight at 90°C and ground in a Wiley mill to pass a 20-mesh screen. One-gram samples of the ground litter were again oven dried at 105°C for 1 hour and weighed. Ash contents were determined by weighing the residue after heating at 550°C for 16 hours.

The respiration rate of the litter samples was measured similarly to the method used by Ruhling and Tyler (22). Fresh litter, equivalent to 2.5 g of oven-dried material, was placed in a 1-liter polycarbonate bottle with sufficient water to attain a 75% moisture content (fresh weight basis). After 1 day of equilibration to ambient laboratory temperature (23 ± 1°C) and the new moisture content (field moisture varied from 27 to 75%), the atmosphere inside the bottle was purged with air for 15 sec by placing a hose, attached to the laboratory vacuum line, near the bottom of the bottle. A small glass vial containing 5 ml of 0.488*N* NaOH was placed inside and the bottle sealed with a plastic screwcap. After 48 hours, the vial was replaced with a second alkali-containing vial after again purging the atmosphere and the incubation continued for a second 48-hour period. The evolved CO_2 in the NaOH was determined by titrating excess alkali, after precipitation of CO_2 by the addition of 2 ml of 1*M* $BaCl_2$, with 0.473*N* HCl using phenolphthalein as an indicator. The acid was standardized with primary standard grade Tris (Sigma Chemical Co.). One in every 10 bottles was a blank without litter. The mean respiration rate for the two 48-hour incubations was expressed as micrograms of CO_2 evolved per gram of over-dried litter per hour.

Enzyme extractions were achieved by transferring the litter from the bottle into a Waring blendor (Model 1120) with 100 ml of 0.05*M* potassium acetate, pH 5.0. The sample was ground for 1 min and the resulting homogenate centrifuged for 15 min at 9,400 g at 2°C in a Sorvall RC-5 refrigerated centrifuge. The supernatant was filtered through Whatman no. 1 paper to remove occasional coarse litter fragments yielding a clear, yellow solution. The extract was stored at 2°C until analyses were completed within 2 days.

Activities of carbohydrase enzymes in this extract were determined by placing 1 ml of substrate solution in 2 ml of extract. The substrate solutions for the assays were as follows: for cellulase, 3% carboxymethylcellulose (CMC), Na salt (Sigma Chemical); for amylase, 6% soluble starch (Baker), solubilized by heating to 95°C; for invertase, 6% sucrose (Difco); and for xylanase, 6% xylan (Aldrich). All substrates were prepared in 0.05*M* potassium acetate, pH 5.0, as solutions except xylan which was a suspension prepared by mixing xylan and buffer in a Waring blendor for 2 min; the suspension was then heated with stirring to 95°C at which point the xylan appears to form a gel. Immediately, the gel was cooled to room temperature and a finely-divided precipitate formed; this was used as the substrate suspension. After incubation (2 hours for xylan or CMC and 4 hours for starch or sucrose) at room temperature (ca. 23°C), the reducing sugar content was determined colorimetrically with dinitrosalicylic acid (4). This strongly alkaline reagent also serves to stop the enzymatic reaction. Blanks, containing extract without substrate and substrate solutions without extract, were run concurrently to correct for background levels of reducing substances. In the xylanase assay, suspended xylan was removed after color development, but prior to optical density determination by centrifuging the sample at 4,000 × *g* for 10 min at 2°C. Activity in all cases was expressed in micromoles of reducing sugar released per oven-dried gram of litter per hour. The background level of reducing substances was calculated in micromoles of reducing sugar per gram of oven-dried litter.

Peroxidase activity was determined by the method of Bartha and Bordeleau (2) except that the buffer was 0.05*M* potassium acetate, pH 5.0, instead of 0.05*M* potassium phosphate, pH 6.0. Activity was expressed as nanomoles of H_2O_2 reduced per oven-dried gram of litter per min.

The effect of heating on the extracted enzymatic activities was tested by placing the extract in a boiling water bath for 15 min and performing the assay as above. The effect of storing an extract at 2°C was examined by assaying extracts at daily intervals from 0 to 3 days. An extracting buffer which would yield relatively high enzymatic activities was selected from several buffers: 0.05*M* potassium acetate, pH 5.0; 0.05*M* potassium citrate, pH 5.0; 0.05*M* potassium phosphate, pH 6.5; 0.05*M* potassium maleate, pH 6.0; and 0.05*M* Tris chloride, pH 7.0. Extractions and assays using these buffers were performed as described above.

The hexose content of the extract was determined using the anthrone reagent (25) with D-glucose (10 to 200 ppm) as a standard. Determinations were expressed in milligrams of hexose per oven-dried gram of litter. The total phenolic content of the extract was measured by the method of Swain and Hillis (28) using *D*-

Table 1—Linear correlation coefficients between biochemical variables of coniferous leaf litter.†

Variable	Respiration	Phenolics	Hexose	Reducing substances	Xylanase	Cellulase	Invertase	Amylase
Phenolics	0.53							
Hexose	0.74	0.72						
Reducing substances	0.73	0.71	0.95					
Xylanase	0.44	0.39	0.47	0.55				
Cellulase	0.58	0.39	0.54	0.61	0.91			
Invertase	NS	NS	NS	NS	NS	NS		
Amylase	0.71	0.38	0.62	0.64	0.62	0.66	0.34	
Peroxidase	NS	0.64	NS	NS	NS	NS	NS	NS

† NS = Not significant; all tabulated coefficients are significant at the 1% level.

(+)-catechin (5 to 50 ppm) as a standard. Results were expressed as micrograms of phenolics equivalent to *D*-catechin per oven-dried gram of litter.

Linear correlation coefficients between all possible pairs of variables were computed for the total of 84 samples. Only coefficients significant at the 1% level are reported. A multiple regression analysis of respiration (dependent variable) on the biochemical variables (independent) was also attempted.

RESULTS

Figure 2 shows the validity of the assays; the rate of hydrolysis of the carbohydrates is a linear function of time for the range of activities encountered in the sample extracts. Peroxidase activity also followed zero-order kinetics and was observed during each assay which involves a continuous colorimetric monitoring of product concentration with time. All carbohydrase and > 90% of the peroxidase activity was destroyed by placing the extracts in a boiling water bath for 15 min. Extracts could be stored at 2°C for up to 3 days with < 10% decline in any carbohydrase activity; peroxidase activity, however, tended to increase with storage. All peroxidase assays were performed at the same time relative to extraction: one day after. The relative enzymatic activities of the several buffered extracts are presented in Table 2.

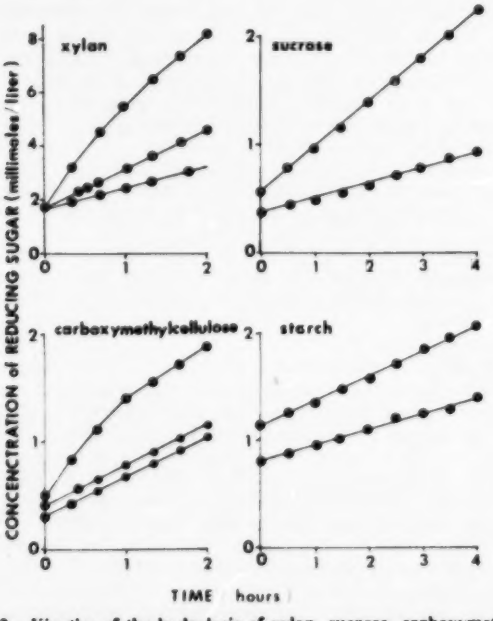

Fig. 2—Kinetics of the hydrolysis of xylan, sucrose, carboxymethylcellulose, and starch by 0.05*M* potassium acetate, pH 5.0, extracts of coniferous leaf litter. The several samples presented represent the range of each enzymatic activity encountered in the sample population.

Table 2—Relative enzymatic activities of buffered extracts of coniferous leaf litter.

Enzymatic activity	Buffer (pH)†				
	Acetate (5.1)	Citrate (5.1)	Maleate (6.1)	Phosphate (6.4)	Tris (6.8)
	% of maximum				
Xylanase	79	99	100	78	66
Cellulase	91	100	75	58	35
Invertase	81	100	54	26	26
Amylase	66	50	63	59	100
Peroxidase	100	nd‡	73	44	16

† All buffers were 0.05*M* as their potassium salts except Tris which was as chloride.
‡ nd = not determined.

The tabulated linear correlation coefficients between paired variables are presented in Table 1. The relationship between respiration and extractable hexose is depicted in Fig. 3; this is almost identical to the relation between respiration and extractable reducing substances since hexose and reducing substances are strongly related (Table 1). Amylase was the enzymatic activity best correlated with respiration and this relationship is presented in Fig. 4. The strong correlation between xylanase and cellulase activity is the most noteable among the biochemical variables (Fig. 5). The mean, range, and variance of each variable in the sample population is presented in Table 3. The regression equations for five of the better correlations not presented in the figures are:

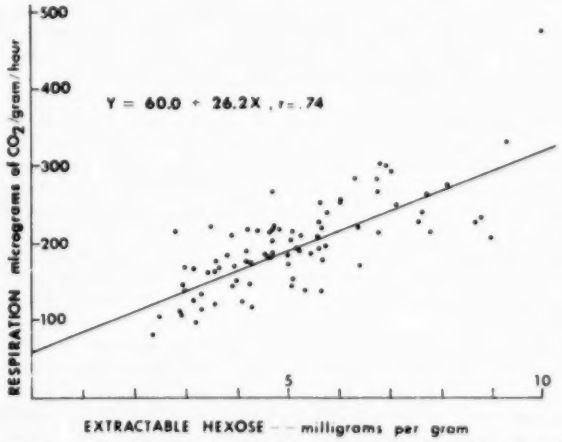

Fig. 3—The relationship between the respiration of samples of coniferous leaf litter and the amount of extractable hexose.

Respiration (*Y*), Extractable Phenolics: $Y = .104X + 124$
Respiration (*Y*), Reducing Substances: $Y = 3.04X + 97.8$
Respiration (*Y*), Xylanase Activity: $Y = .647X + 145$
Respiration (*Y*), Cellulase Activity: $Y = 3.89X + 92.9$
Reducing Substances (*Y*), Extractable Hexose: $Y = 8.11X - 9.84$

Multiple regression of respiration on extractable hexose, phenolics, and reducing substances did not significantly improve the correlation of respiration on extractable hexose alone. Multiple regression of respiration on all five enzymatic activities did not provide a significant reduction in residual sum of squares compared with regression on amylase activity alone. The expression of all variables on an ash-free basis did not change any correlation significantly even though the ash values varied widely (Table 3).

DISCUSSION

If the decomposition of plant litter is achieved through the agency of extracellular enzymes, then these enzymes should be both extractable and, more importantly, related to the rate of decomposition within litter of a given type with similar abiotic factors. In this study, extractable enzymatic activities including cellulase, xylanase, and amylase were correlated with the average rate of coniferous leaf litter respiration. The correlation with cellulase and xylanase might have been expected since both cellulose and hemicellulose are the major constituents of the coniferous leaf litter examined (29). Microorganisms from composting leaves have been shown to produce extracellular cellulase and amylase activities in vitro (9), but cellulase activity has been little studied in vivo. Stutzenberger et al. (27) observed a significant relation between extractable cellulolytic activity and cellulose decomposition in composting municipal solid waste. Xylanase in vivo has received even less attention although the ability of wood-rotting microorganisms to produce xylanase in vitro is quite well established (13). The strong correlation between xylanase and cellulase (Fig. 5) is not surprising since, in vitro, several fungi produce the two enzymes which are distinct since they behave differently during fractionation (1). The decomposition of cellulose and hemicellulose seems to proceed simultaneously with many wood-rotting fungi (13). The strong correlation observed here would preclude the necessity of assaying both activities in future studies with coniferous leaf litter.

Table 3—Mean, range, and variance of the biochemical variables of extracts of coniferous leaf litter.

Variable	Units per gram of ovendry litter	Mean	Range	Variance†
Respiration	micrograms of CO_2/hour	194	79–470	3,573
Phenolics	micrograms	685	340–2,447	92,500
Hexose	milligrams	5.17	2.37–10.04	2.877
Reducing substances	micromoles	32.1	6.8–74.9	209.1
Xylanase	micromoles released/hour	77.9	24.5–207.0	1,658
Cellulase	micromoles released/hour	26.4	12.9–52.9	79.22
Invertase	micromoles released/hour	10.0	3.4–33.7	28.04
Amylase	micromoles released/hour	10.4	4.2–23.5	17.86
Peroxidase	nanomoles of H_2O_2 reduced per minute	1,463	195–19,980	5,649,000
Ash	grams	0.144	0.026–0.406	0.008446

† Variance = $\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N}$, where $N = 84$.

The relationship between amylase activity and the rate of decomposition is not likely causal since coniferous leaf litter contains little, if any, starch (16). Nonetheless, Ross and Roberts (20) did observe a strong relation between respiration and amylase activity in tussock grassland soils. However, they did not observe this relationship in *Nothofagus truncata* litter (21). Many fungi utilize glycogen as a storage polysaccharide (6) and a level of amylase activity proportional to the size of the fungal population might be necessary for cell constituent turnover. Thus, the correlation between amylase activity and the rate of decomposition might reflect the size of the fungal decomposer population.

The only correlation with invertase activity was amylase activity albeit a rather weak one (Table 1). Ross and Roberts (20) and Spalding et al. (24) observed a strong relation between invertase activity and respiration in various soils. However, Ross and Roberts (21) observed poor correlation between invertase activity and O_2 uptake in *Nothofagus truncata* litter. Invertase activity probably reflects a physiological trait of some fraction of the microbial flora in decomposing coniferous litter which might be a dominant fraction in certain soils. Likewise, peroxidase activity was unrelated to respiration or any other enzymatic activity; it was, however, correlated with the level of extractable phenolics which is consistent with its suspected role in lignin

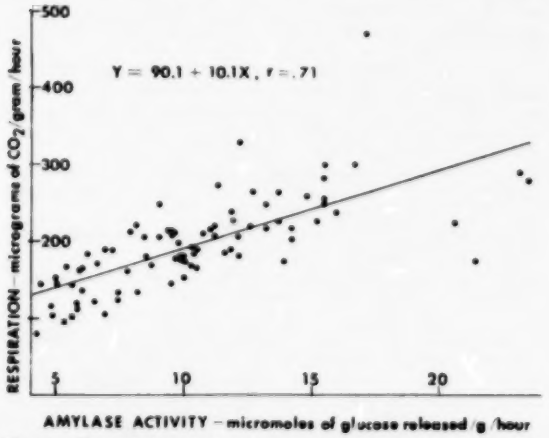

Fig. 4—The relationship between the respiration of samples of coniferous leaf litter and the extractable amylase activity.

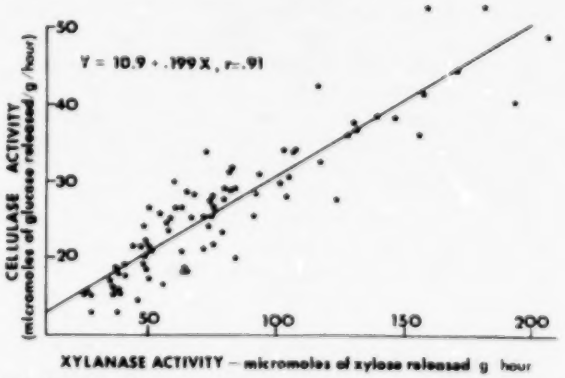

Fig. 5—The relationship between cellulase and xylanase activities in coniferous leaf litter extracts.

decomposition (12). Niether invertase nor peroxidase are likely involved in any rate limiting process maintaining a pool of readily metabolizable substances as depicted in Fig. 1.

The rapidity with which the respiration of soil (15) and litter (21, 30) responds to the addition of glucose or other simple molecules would suggest that these compounds are limiting microbial respiration. In the present study, a pool of readily metabolizable substances including extractable hexose, phenolics, and reducing substances was correlated with the rate of decomposition (Table 1). The exact chemical identity of these extractable substances is not known, but two chemically independent methods, reducing power and anthrone complexation, are strongly related ($r = 0.95$) which suggests that these substances are simple sugars. Ross (19) found that O_2 uptake was markedly influenced by water soluble carbohydrates in *Nothofagus truncata* litter. Similarly, Dormaar (7) observed a decrease in water soluble hexose and phenolics in *Populus* leaves before and after leaf fall. In soils, hexose (anthrone method) extractable in water or mild salt solutions has been correlated with a variety of microbial processes including mineralization potential (11) and denitrification rate (26). Hayes (10) observed a rapid decrease in extractable polyphenol content of coniferous leaf litter during decomposition. Shindo and Kuwatsuka (23) observed a similar decline in extractable phenolics during the decay of rice straw. In the present study, extractable phenolics were correlated with the rate of decomposition and are likely part of the pool of readily metabolizable substrates in the litter.

The correlation between enzymatic activities and extractable substrates is suggestive of a cause/effect relation. The basis for such a relation is depicted in the conceptual model of plant litter decomposition (Fig. 1). Chang (5) observed a good relationship between weight loss and disappearance of hemicellulose and cellulose in wheat straw compost which would suggest the importance of enzymes degrading these fractions. The utility of the model in Fig. 1 in predicting relationships among variables during plant litter decomposition is substantiated by the present observations. More of the residual variability in the instantaneous rate of litter decomposition might be explained by measuring additional enzymatic activities including mannase, polyphenol oxidase, and protease. Levels of extractable amino acids might also be related to the rate of decomposition. Substrate composition would strongly influence decomposition and measurements of the amounts of cellulose, hemicellulose, lignin, and protein by proximate analysis or detergent extraction (31) should prove useful.

Litter decomposition is, of course, an intregal part of all ecosystems, but the realization of the importance of understanding its processses, to both model and understand whole ecosystems, is increasing(18). In order to understand litter decomposition, it will be necessary to do more than passively observe the process as has been done in the present work; the system must be perturbed and changes in suspected causal variables observed. For instance, heavy metals are known to inhibit litter decomposition (22). Although decomposer organisms likely have mechanisms to exclude heavy metals intracellularly, microbial growth in litter is limited by the release of assimilable substrate which proceeds in the extracellular milieu containing these heavy metals. Extracellular enzymes may or may not function at certain levels of such pollutant stress; this can be tested. However, just which enzymatic activities to observe is not a simple question. It is the purpose of the present effort to find out which enzymatic activities are related to coniferous leaf litter decomposition and use these observations to select variables for study when litter decomposition is perturbed by pollutant or environmental stresses.

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